Microbiology (2014), **160**, 1523–1532 DOI 10.1099/mic.0.078477-0

Identification of a novel ATP-binding cassette transporter involved in long-chain fatty acid import and its role in triacylglycerol accumulation in *Rhodococcus jostii* RHA1

María S. Villalba[1,2] and Héctor M. Alvarez[1]

Correspondence
Héctor M. Alvarez
halvarez@unpata.edu.ar

[1]Centro Regional de Investigación y Desarrollo Científico Tecnológico, Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia, San Juan Bosco Km 4-Ciudad Universitaria 9000, Comodoro Rivadavia (Chubut), Argentina

[2]Oil m&s, Av. Hipólito Yrigoyen 4250, 9000 Comodoro Rivadavia (Chubut), Argentina

Members of the genus *Rhodococcus* are specialists in the biosynthesis and accumulation of triacylglycerols (TAGs). As no transport protein related to TAG metabolism has yet been characterized in these bacteria, we used the available genomic information of *Rhodococcus jostii* RHA1 to perform a broad survey of genes coding for putative lipid transporter proteins in this oleaginous micro-organism. Among the seven genes encoding putative lipid transporters, *ro05645* (now called *ltp1*: lipid transporter protein) coding for an ATP-binding cassette protein was found clustered with others genes encoding enzymes catalysing the three putative acylation reactions of the Kennedy pathway for TAG synthesis. Overexpression of *ltp1* in the RHA1 strain led to an increase of approximately sixfold and threefold in biomass and TAG production, respectively, when cells were cultivated on palmitic acid and oleic acid. Moreover, overexpression of *ltp1* also promoted a significant increase in the uptake of a fluorescently labelled long-chain fatty acid (LCFA), as compared with the WT strain RHA1, and its further incorporation into the TAG fraction. Gluconate-grown cells showed increasing amounts of intracellular free fatty acids, but not of TAG, after overexpressing *ltp1*. Thus, for the first time to our knowledge, a transporter functionally related to TAG metabolism was identified in oleaginous rhodococci. Our results suggested that Ltp1 is an importer of LCFAs that plays a functional role in lipid homeostasis of *R. jostii* RHA1.

Received 20 February 2014
Accepted 11 April 2014

INTRODUCTION

In recent years, the genus *Rhodococcus* has been studied extensively due to its potential biotechnological applications. These metabolically versatile actinobacteria possess interesting physiological capabilities, such as their capacity for degrading a diversity of pollutants, their ability to adapt to diverse environments and their wide anabolic potential (Warhurst & Fewson, 1994; LeBlanc *et al.*, 2008; Marqués *et al.*, 2009). In this context, one interesting feature of rhodococci is their ability to produce high amounts of lipids, such as triacylglycerols (TAGs) (Alvarez & Steinbüchel, 2002; Alvarez *et al.*, 2013). Some members of these actinobacteria can be considered oleaginous micro-organisms since they are able to accumulate significant amounts of TAGs after cultivation of cells under nitrogen-limiting conditions (Alvarez *et al.*, 1996).

The biosynthesis and accumulation of TAGs by rhodococci have gained increasing interest due to the potential applications of lipids in the biofuel industry. More recently, advances in the molecular biology and genetics of TAG metabolism have occurred as a result of the increased availability of genomic information and the development of new genetic tools for rhodococci (Holder *et al.*, 2011; Chen *et al.*, 2013; Villalba *et al.*, 2013). These studies have been focused principally on some enzymes of TAG biosynthesis, such as the wax esters/diacylglycerol acyltransferases (WS/DGAT) (Alvarez *et al.*, 2008; Hernández *et al.*, 2013), NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase (MacEachran & Sinskey, 2013), and phosphatidic acid phosphatase (M. A. Hernández, A. Arabolaza, S. Comba, H. Gramajo & H. M. Alvarez, unpublished). Moreover, some genes coding for TAG granule-associated proteins have been identified and characterized in *Rhodococcus opacus* and *Rhodococcus jostii* (MacEachran *et al.*,

Abbreviations: ABC, ATP-binding cassette; DAG, diacylglycerol; FFA, free fatty acid; LCFA, long-chain fatty acid; NCBI, National Center for Biotechnology Information; TAG, triacylglycerol.

Three supplementary tables and two supplementary figures are available with the online version of this paper.

2010; Ding *et al.*, 2012; Chen *et al.*, 2013). In addition to these enzymes and structural components of lipid inclusions, other relevant proteins involved in TAG metabolism, such as transporters and transcriptional regulators, remain to be identified. In this context, the involvement of lipid transporters in the maintenance of lipid homeostasis in eukaryotic cells has been established (Dean *et al.*, 2001; Pohl *et al.*, 2005; Nagao *et al.*, 2010).

In particular, MDR1 (1280 aa) is a well-characterized lipid transporter occurring in human cells which is responsible for multidrug resistance and is able to export a wide variety of substances, most of them hydrophobic or amphiphilic unrelated compounds (Borst *et al.*, 2000; Dassa & Bouige, 2001; Dean *et al.*, 2001; Nagao *et al.*, 2010). This efflux pump belongs to the ATP-binding cassette (ABC) superfamily of transporters and consists of two halves, each half including a hydrophobic transmembrane region or transmembrane domain with six putative α helices and a nucleotide-binding domain; these two halves cooperate to form a single full transporter. Through ATP hydrolysis, this protein has the ability to extrude several amphiphilic compounds from cells against a substrate concentration gradient (van Veen *et al.*, 1996, 2006). Homologues to MDR1 are found almost exclusively in eukaryotes, although they have also been found and characterized in some prokaryotes, e.g. MsbA in *Escherichia coli* and Sav1866 in *Staphylococcus aureus* (Zhou *et al.*, 1998; Borst *et al.*, 2000; Davidson & Chen, 2004; Velamakanni *et al.*, 2008). These two transporters are homodimers made up of half-size transporters or monomers that consist of a transmembrane domain fused to a nucleotide-binding domain. In particular, the MsbA protein (582 aa) plays a major role in *E. coli* physiology as it functions as a protein that transports lipid A, glycerophospholipids and other non-specific hydrophobic compounds (Eckford & Sharom, 2008; Kaul & Pattan, 2011). In fact, *msb*A has been proposed as an essential gene as some defects or mutations on this gene cause accumulation of lipid A and phospholipids in the inner membrane, which is lethal for *E. coli* (Zhou *et al.*, 1998). Interestingly, Doshi *et al.* (2013) proposed the overexpression of the MsbA protein transporter for excreting isoprenoids and other commercial compounds in *E. coli* for biotechnological purposes. Sav1866 of *S. aureus* is also considered as a lipid transporter homologous to MsbA and MDR1 proteins, which mediates active extrusion of multiple hydrophobic drugs (Velamakanni *et al.*, 2008). Recently, some transporters belonging to the ABC superfamily involved in the uptake and catabolism of several cholate metabolites, as well as phthalate and phthalate esters, have been reported for *R. jostii* RHA1 (Swain *et al.*, 2012; Hara *et al.*, 2010). However, none of these proteins seems to be related to TAG metabolism. Despite the obvious importance of lipid transporter proteins in TAG metabolism and lipid homeostasis in oleaginous rhodococci, none has yet been characterized. For these reasons, we performed a broad survey of genes coding for putative lipid transporters in the *R. jostii* RHA1 genome, and identified and functionally characterized an ABC transporter involved in the provision of fatty acids to TAG metabolism. The identification of a new gene related to TAG metabolism in oleaginous rhodococci will contribute to a better understanding of these industrially relevant micro-organisms.

METHODS

Bacterial strains, plasmids, media and growth conditions. The strains and plasmids used in this work are listed in Table S1 (available in the online Supplementary Material). *E. coli* strains were grown on solid or liquid LB medium at 37 °C. *R. jostii* strain RHA1 and its recombinant were grown aerobically at 28 °C in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989). To analyse growth, cells were grown in solidified mineral salts medium (MSM) according to Schlegel *et al.* (1961) with 1 g NH_4Cl l^{-1} (MSM1; nitrogen rich), and with sodium gluconate 1 % (w/v), tripalmitin, olive oil, palmitic acid and oleic acid 0.5 % (v/v) as carbon sources, and plates were incubated for 7–10 days. To promote accumulation of lipids, the concentration of NH_4Cl in MSM was reduced to 0.1 g l^{-1} (MSM0.1; nitrogen-limiting conditions). Sodium gluconate 1 % (w/v), tripalmitin 0.025 % (w/v), oleic acid and olive oil 0.3 % (v/v), and palmitic acid 0.1 % (w/v) were used as a sole carbon sources, respectively. Cells were harvested at specific time points, washed with a saline solution [NaCl 0.85 % (w/v)] and dried for chemical analyses. If necessary, the antibiotics ampicillin and kanamycin were used at final concentrations of 100 and 50 μg ml^{-1}, respectively. Induction of the acetamidase (P_{ace}) promoter of pJAM2 and its derivatives was routinely achieved by addition of 0.5 % (w/v) acetamide to the respective cultures.

Analyses of sequences. MsbA (from *E. coli* K-12 substrain MG1655; GenBank accession number NP_415434.1) and Sav1866 (from *S. aureus* subsp. *aureus* Mu50; GenBank accession number NP_372390.1) protein sequences were downloaded from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/); these sequences were used as queries for further searches using the BLASTP program from the NCBI website in order to find homologous proteins in *R. jostii* RHA1. The search was run using the default parameters set by the program, considering as homologous proteins those with significant alignments (*E* value: 10^{-48}). Additionally, in order to determine the occurrence of proteins homologous to RO05645 in other rhodococcal species, its sequence was used to perform a BLAST search of NCBI genomic databases; and in the case of *Rhodococcus fascians* F7, also from the RAST (Aziz *et al.*, 2008) server. In addition, we analysed the genomic contexts for all homologous putative transporters found in *Rhodococcus* species from the NCBI website. Multiple alignments were performed using BioEdit Sequence Alignment Editor. We used the Philius Transmembrane Prediction Server (http://www.yeastrc.org/philius/) to establish a secondary structure prediction from protein sequences.

DNA analysis, amplification, cloning, sequencing and transfer by electroporation. Chromosomal DNA, plasmids and DNA fragments were isolated and analysed by standard methods (Marmur, 1961; Sambrook *et al.*, 1989). For DNA amplification of complete ORFs, PCR was performed using specific primers (Table S2). The thermocycling parameters were: 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 30 s at 65 °C, 2 min 20 s at 72 °C and finally 5 min at 72 °C. The PCR products were cloned into pGEM-T Easy vector and subjected to sequencing. Plasmid derivatives were introduced in *Rhodococcus* strains by electroporation. Assays were performed using a model 2510 electroporator (Eppendorf-Netheler-Hinz) and transformation was carried as described by Kalscheuer *et al.* (1999). To obtain recombinant strains, 400 μl aliquots of competent cells were

mixed with DNA, preincubated at 40 °C for 5 min followed by 10 min of incubation on ice and then electroporated.

Cloning and overexpression. Once selected by high identity with canonical lipid transporters, the *ro05645* gene was amplified from total genomic DNA of *R. jostii* RHA1 by PCR using the primers FRHA1 and RRHA1 (Table S2). Resulting PCR products were cloned in pGEM-T Easy vector (pGEM-T-easy/*ro05645*, Table S1) and verified by DNA sequencing. For overexpression, a *Bam*HI/*Xba*I fragment of pGEM-T-easy/*ro05645* was subcloned into a *Bam*HI/*Xba*I site of the shuttle *E. coli–Mycobacterium–Rhodococcus* vector pJAM2, which contains an inducible acetamide promoter (P_{ace}), and the resulting derivative plasmid pJAM2/*ro05645* was transferred into *R. jostii* RHA1 WT cells by electroporation as reported previously (Hernández *et al.*, 2013).

Generation of a single *ro05645* disrupted mutant of *R. jostii* RHA1. To disrupt the *ro05645* gene, we utilized a strategy reported previously by van der Geize *et al.* (2000) and Hernández *et al.* (2013), using a derivative of the pGEM-T Easy vector as a suicide plasmid. For this, an internal fragment of 763 bp of the *ro05645* gene was amplified by PCR using primers F3RHA1 and R3RHA1 (Table S2), and cloned into the pGEM-T Easy vector to get the plasmid pGEM-T-easy/'*ro05645*'. Then, a kanamycin-resistant cassette obtained from the plasmid pUC4K was cloned into the *Bam*HI restriction site of pGEM-T-easy/'*ro05645*' to get pGEM-T-easy/'*ro05645*'-ΩKm. The resulting suicide plasmid was transferred to *R. jostii* RHA1 by electroporation as reported by Hernández *et al.* (2013).

BODIPY FL C_{16}-labelled fatty acid uptake. Cells were grown aerobically for 24 h at 28 °C in LB medium, collected by centrifugation, washed three times in a saline solution [NaCl 0.85 % (w/v)] and resuspended in 20 ml MSM0.1. Both media were supplemented with kanamycin and acetamide at final concentrations of 50 μg ml^{-1} and 0.5 % (w/v), respectively. The fluorescent long-chain fatty acid (LCFA) analogue BODIPY FL C_{16} (D-3821; Molecular Probes) was added from a 5 mM ethanolic stock solution to 20 ml MSM0.1 cultures to a final concentration of 5 μM and these cultures were incubated aerobically at 28 °C for 10 h. A culture sample of 5 ml was taken after 4 h of incubation, centrifuged, filtered through 0.22 μm polyethersulfone filters (Gamafil), and the supernatants were used to measure fluorescence intensity and OD_{600}. Fluorescence measurements were performed using 10 mm × 10 mm cuvettes in a JASCO FP-6200 spectrofluorometer; samples were excited at 496 nm and the emission was recorded at 509 nm. OD_{600} was also measured in 10 mm × 10 mm cuvettes in an SP-1103 spectrophotometer. All spectral measurements were done in triplicate. Finally, a second sample was taken after 10 h of incubation in order to analyse the TAG content of cells by semiquantitative TLC.

Lipid analysis. The qualitative and semiquantitative analyses of intracellular lipids in *Rhodococcus* strains were performed by TLC. For intracellular analysis, 4–5 mg lyophilized cells were extracted with chloroform/methanol (2 : 1, v/v) for 120 min at 4 °C. Between 15 and 30 μl extracts (depending on culture conditions) were separated by TLC, which was performed on silica gel 60F254 plates (Merck) using hexane/diethyl ether/acetic acid (80 : 20 : 1, v/v/v) as mobile phase (Wältermann *et al.*, 2000). Tripalmitin (Fluka) and oleic acid (Fluka) were used as lipid reference substances. Lipid fractions were visualized after brief exposure to iodine vapour or UV light.

To determine the fatty acid content of the cell and the composition of lipids, 5–10 mg dried whole cells was subjected to methanolysis in the presence of 15 % (v/v) sulfuric acid as described by Brandl *et al.* (1988), and the resulting acyl-methylesters were analysed by GC using an HP 5890A gas chromatograph equipped with a Factor Four capillary columnVF-23ms (30; 0.25; 0.25) and a flame ionization detector. The injection volume was 0.2 ml and helium (13 mm min^{-1}) was used as carrier gas. A temperature programme was used for efficient separation of the methyl esters (80 °C 1 min, an initial ramp of 10 °C min^{-1} up to 160 °C, then an increase of 3 °C min^{-1} up to 200 °C and a final ramp of 30 °C min^{-1} up to 240 °C maintained for 5 min to allow column cleaning). For quantitative analysis, tridecanoic acid was used as an internal standard.

Antibiotic sensitivity assay. To establish antibiotic sensitivity/resistance for the WT and recombinant strains, we used the disc diffusion method reported by Jorgensen & Ferraro (2009). Briefly, cell suspensions of OD_{600} 0.2 were spread onto the surface of LB agar plates (150 mm diameter). Commercially available multidiscs (Britania) embedded with different antibiotics were placed on the inoculated agar surface. Plates were incubated for 48–72 h at 28 °C and the zones of growth inhibition formed around each disc were analysed. The following antibiotics were included: norfloxacin (10 μg), gentamicin (10 μg), cefalotin (30 μg), nitrofurantoin (300 μg), ampicillin/sulbactam (10/10 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg).

RESULTS

Bioinformatic analyses of genes coding for putative lipid transport proteins

We used *E. coli* MsbA and *S. aureus* Sav1866 protein sequences to screen the *R. jostii* RHA1 genome for proteins involved in lipid transport. BLASTP analyses identified several putative ABC transporters with relatively high identities to Sav1866 in the RHA1 genome (Table 1). Two of the identified proteins (RO04958 and RO06013) presented an amino acid length similar to MDR1 (1280 aa), whereas the rest of the proteins exhibited an amino acid length between 577 and 681 aa, similar to Sav1866 (578 aa).

All putative transporters of short amino acid length found in RHA1 exhibited highly conserved motifs reported for ABC transporters (Fig. S1). Three conserved motifs are usually found in ABC transporters: (1) the Walker A motif consisting of GXXGXGKS/T, where X represents any amino acid, (2) the Walker B motif containing ΦΦΦΦD, where Φ is any hydrophobic residue, and (3) the C loop (LSGGQ), called the signature motif or linker peptide, which is unique to ABC transporter proteins (Schneider & Hunke, 1998). As all these putative transporters found in the RHA1 genome possessed these three conserved motifs and similar amino acid lengths, they may be considered as putative half-sized ABC transporters, similar to Sav1866 and MsbA (Fig. S1).

In addition to BLASTP analyses, we investigated the genomic organization for all half-sized transporters found in *R. jostii* RHA1. Interestingly, the *ro05645* gene was localized in a cluster containing genes encoding putative proteins involved in TAG and phospholipid metabolism (Fig. 1), whereas the rest of the analysed genes were organized randomly in the RHA1 genome (data not shown). For this reason, we decided to investigate protein RO05645 in more detail, analysing its probable folding organization into the plasma membrane in comparison with Sav1866 and MsbA (Fig. S2). The secondary structure predictions of the three proteins

Table 1. Proteins homologous to Sav1866 identified from the *R. jostii* RHA1 genome database

Database entry	Enzyme name	Length (aa)	GenBank accession no	Amino acid identity (%)
RO04169	Bifunctional lipid A exporter	577	YP_704120	28
RO04170	Bifunctional lipid A exporter	659	YP_704121	33
RO04711	ABC bifunctional lipid A exporter	582	YP_704655	27
RO04958	Bifunctional ABC multidrug transporter	1278	YP_704897	35
RO05645	Bifunctional lipid A transporter	681	YP_705582	32
RO06013	Multidrug ABC transporter	1306	YP_705948	34
RO06043	Bifunctional ABC lipid A transporter	632	YP_705978	30

showed almost identical profiles with no identified signal peptide, and six transmembrane helices and six cytoplasmic portions located in similar amino acids positions.

In addition, we searched the genome of other rhodococcal species for the occurrence of orthologues of *ro05645*. Interestingly, these genes were found uniquely in those species considered as oleaginous rhodococci, such as *R. opacus* and *Rhodococcus wratislaviensis*; whereas in *Rhodococcus erythropolis*, *Rhodococcus equi* and *R. fascians*, which usually accumulate lower amounts of TAG from gluconate or glucose, these genes were absent (Table S3). Orthologues of *R. opacus* and *R. wratislaviensis* were also localized in the same lipid cluster as in *R. jostii* RHA1.

Role of *ltp1* in biomass production and TAG accumulation

In order to analyse the *in vivo* role of *ro05645* (here called *ltp1*: lipid transporter protein) in cell growth and TAG accumulation by *R. jostii* RHA1, we performed knockout mutagenesis and overexpression analyses. To delete *ltp1*, we used a single recombination strategy, which was applied successfully in previous mutagenesis analyses in *R. opacus* PD630 (Hernández *et al.*, 2013). After nine consecutive electroporation attempts using the suicide plasmid pGEM-T-easy/'*ro05645*'-ΩKm, we failed repeatedly to disrupt *ltp1* and no mutant strains were obtained. However, overexpression of *ltp1* in *R. jostii* RHA1 was achieved after cloning the gene in the pJAM2 vector under an acetamide-inducible promoter. The recombinant and the WT strain containing the empty vector were analysed for their growth in nitrogen-rich medium (MSM1); while biomass and TAG production were investigated during cultivation in nitrogen-poor mineral medium (MSM0.1). Different substrates, such as gluconate, olive oil, tripalmitin, oleic acid and palmitic acid, were used as sole carbon sources during growth experiments.

The overexpression of *ltp1* promoted an increase of cell growth after cultivation on all lipophilic substrates used in

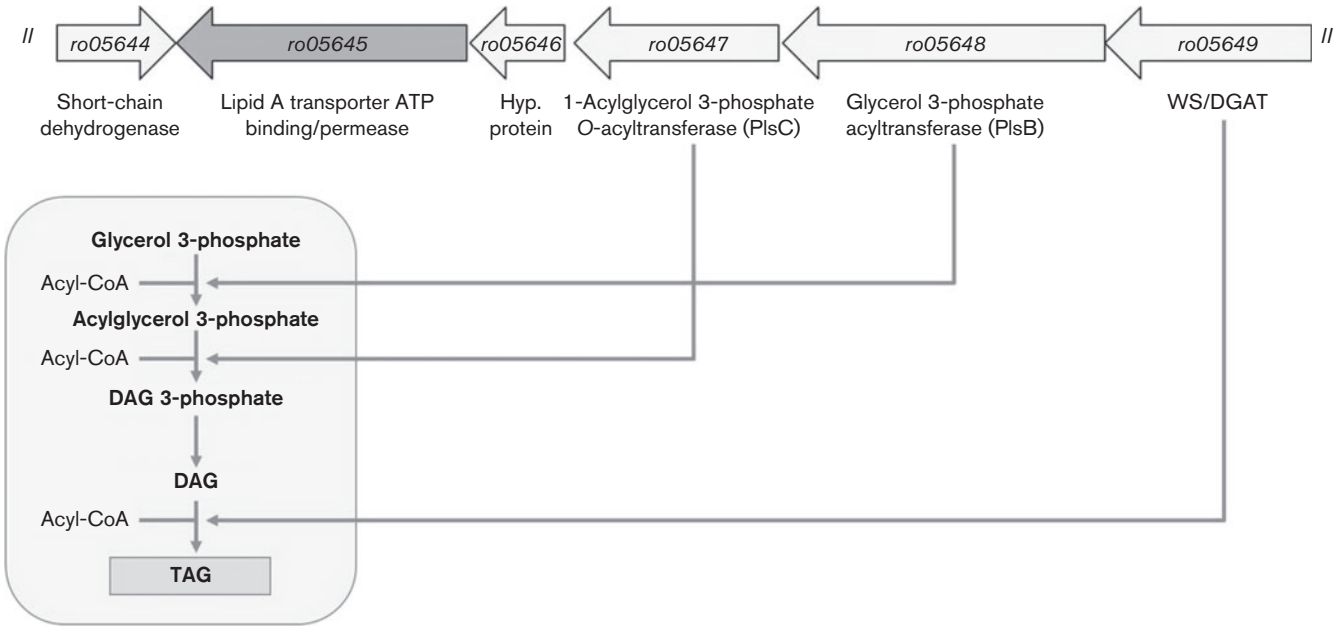

Fig. 1. Localization of the *ro05645* gene (*ltp1*) together with other genes involved in TAG and phospholipid metabolism. Hyp., hypothetical; WS/GGAT, wax ester/diacylglycerol (DAG) acyltransferase.

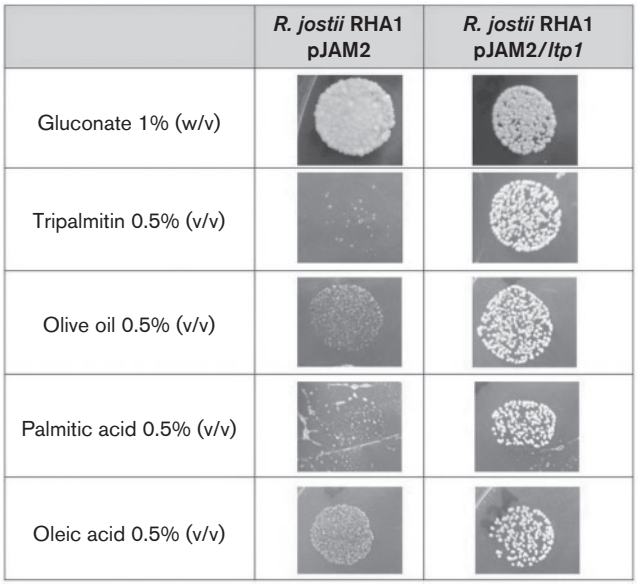

Fig. 2. Growth of *R. jostii* RHA1 strains during cultivation on gluconate and lipophilic substrates as sole carbon sources.

this study, but not on gluconate, as sole carbon sources (Fig. 2). In addition, TLC analyses of the overexpressing strain revealed an increase in TAG production after the induction of *ltp1* in strain RHA1 when cells were cultivated in MSM0.1 with olive oil, tripalmitin, oleic acid and palmitic acid as sole carbon sources (Fig. 3). In contrast, after growth on gluconate as the sole carbon source, *ltp1*-overexpressing cells accumulated increased amounts of free fatty acids (FFAs) with a slight decrease of TAG content, in comparison with the WT strain (Fig. 3). Quantification of lipids showed an increase in total fatty acids (which is a good estimation of TAG content) of more than threefold compared with the WT, and also promoted an increase of cellular biomass production of approximately five and sixfold during cultivation of cells on palmitic and oleic acid as sole carbon sources (Table 2). In contrast, after cultivation of *ltp1*-overexpressing cells on gluconate as the sole carbon source, no increase of TAG and biomass production was detected under the conditions used in this study (Table 2).

Analysis of fatty acid composition revealed no differences between the WT and *ltp1*-overexpressing cells, with the exception of palmitic acid-grown cells of the recombinant strain which showed a slight increase in the relative proportion of palmitic acid ($C_{16:0}$) in their lipids (data not shown).

In order to determine the role of Ltp1 protein on the eventual transport of alternative compounds, such as antimicrobial substances with different chemical structures, we analysed the changes in resistance/sensitivity of recombinant and WT cells to different bioactive compounds, such as norfloxacin, gentamicin, cefalotin, nitrofurantoin, ampicillin/sulbactam and trimethoprim/sulfamethoxazole. We detected no differential responses of cells overexpressing *ltp1* to all tested compounds in comparison with the WT (data not shown).

Effect of the overexpression of *ltp1* on fluorescently labelled fatty acid uptake

With the aim to establish the physiological role of the protein in LCFA uptake by *R. jostii* RHA1 pJAM2/*ltp1*, we

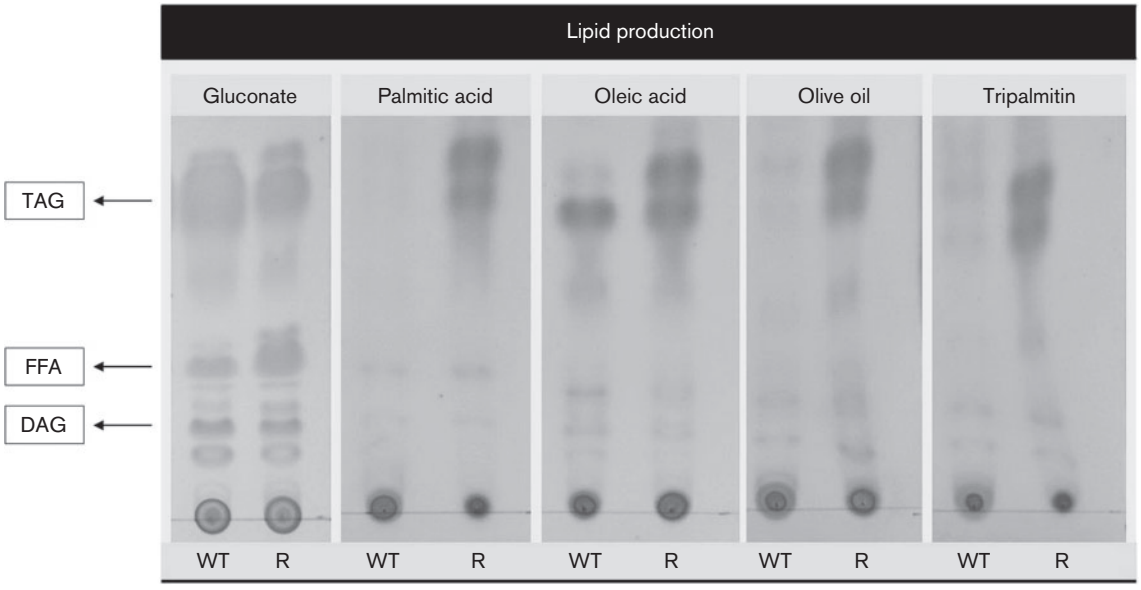

Fig. 3. Lipid analysis of whole-cell extracts of *R. jostii* RHA1 pJAM2 (WT) and *R. jostii* RHA1 pJAM2/*ltp1* (R) strains grown in MSM0.1 with different substrates as carbon sources.

Table 2. Biomass and TAG production in *R. jostii* RHA1 pJAM2 and *R. jostii* RHA1 pJAM2/*ltp1*

R. jostii RHA1	Gluconate 1 % (w/v)		Palmitic acid 0.1 % (w/v)		Oleic acid 0.3 % (v/v)	
	Total fatty acid content (% CDW)	Biomass yield (mg)	Total fatty acid content (% CDW)	Biomass yield (mg)	Total fatty acid content (% CDW)	Biomass yield (mg)
pJAM2	27.4	91.3	7.5	5.9	9.4	7.3
pJAM2/*ltp1*	18.1	85.0	28.2	32.2	32.6	47.2

CDW, cellular dry weight.

analysed the incorporation of a fluorescently labelled LCFA analogue (BODIPY FL C_{16}), using a spectrofluorometric method and TLC analysis. After incubating the cultures for 4 h under nitrogen-limiting conditions (MSM0.1), the *ltp1*-overexpressing strain showed eightfold reduced fluorescence intensity (OD_{600}) in supernatant cultures compared with the WT strain (Fig. 4a), suggesting increased uptake of labelled fatty acid in cells containing higher numbers of transporter proteins in their membranes. Furthermore, intracellular lipids were analysed by TLC, and revealed through UV light and iodine vapour after 10 h of cell incubation (Fig. 4b). Interestingly, *ltp1*-overexpressing cells were able to incorporate higher amounts of labelled fatty acid than WT cells (Fig. 4a). On the other hand, the amounts of FFAs and TAGs were increased in the recombinant strain, whereas an increase of phospholipid and DAG fractions was not observed (Fig. 4b).

DISCUSSION

Despite the importance of lipid transporters in cellular lipid metabolism, the role of such proteins in TAG metabolism of oleaginous rhodococci has not yet been investigated thoroughly. In the present study, we identified a gene coding for a lipid transporter (here called *ltp1*) in *R. jostii* RHA1. Overexpression of *ltp1* in strain RHA1 promoted a significant increase in growth and biomass production when cells were cultivated on lipophilic substrates, such as palmitic acid, oleic acid, tripalmitin and olive oil, but not on gluconate. These results suggested

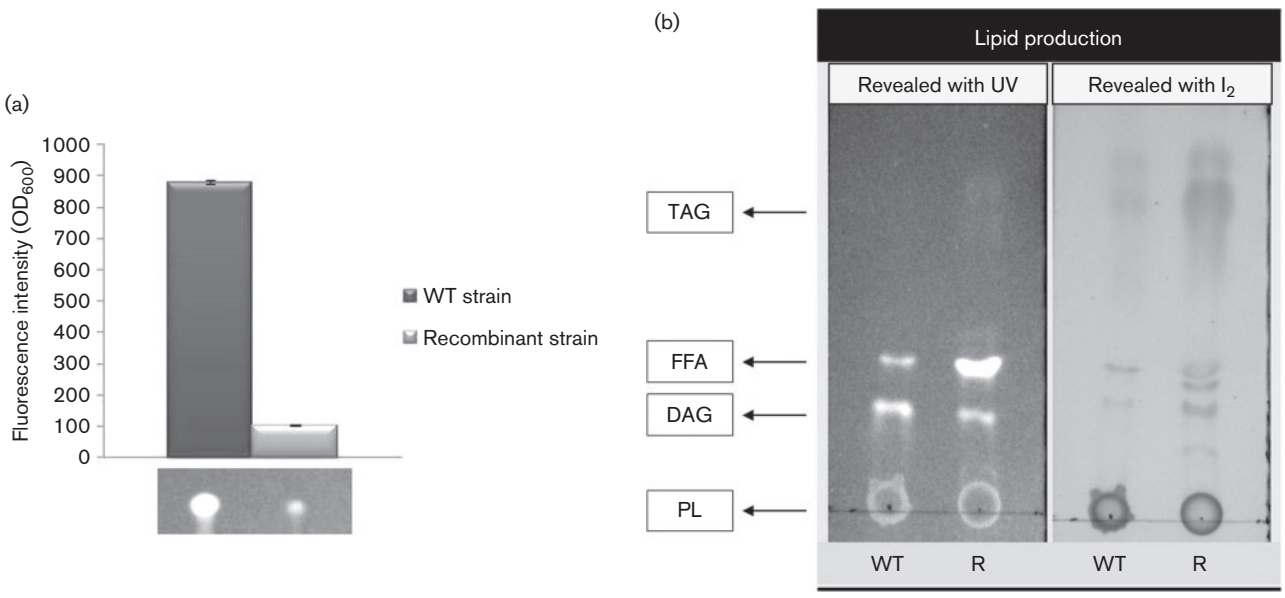

Fig. 4. Fluorescent fatty acid analogue utilization by *R. jostii* RHA1 strains under nitrogen-limiting conditions. (a) Fluorescent measurement of culture supernatants after 4 h of cell incubation (upper panel), and semiquantitative TLC analysis of fluorescent fatty acids extracted from culture supernatants and revealed with UV light (lower panel). (b) Lipid analysis by TLC of intracellular glycerolipids extracted after 10 h of incubation. WT, wild-type strain; R, recombinant strain (RHA1 pJAM2/*ltp1*).

that this transporter protein may function as an 'importer' of hydrophobic compounds in strain RHA1. In contrast to MDR1 and MsbA transporters, which may act as drug importers or exporters, Ltp1 seemed to be more specific for lipid-related compounds. Ltp1 transporter may contribute to the uptake of FFAs or of those fatty acids released by extracellular lipolysis from tripalmitin and olive oil by RHA1 cells. In correlation with this hypothesis, *R. jostii* RHA1 possesses at least 24 genes coding for putative extracellular lipases, according to a previous study (Villalba *et al.*, 2013).

In order to confirm the role of Ltp1 as a fatty acid importer in strain RHA1, we investigated the effect of *ltp1* overexpression on the uptake of a fluorescently labelled LCFA analogue. The overexpression of the transporter promoted a significant increase of the labelled fatty acid uptake and especially its incorporation in the TAG fraction, confirming its role as a fatty acid translocator. Several fatty acid transporter proteins have been characterized so far in both prokaryotes and eukaryotes. In this context, the protein FadL, which mediates LCFA uptake through an active transport in *E. coli*, has been well characterized (Maloy *et al.*, 1981). This transport system seems to be present in other Gram-negative bacteria and probably in some Gram-positive micro-organisms, such as *Mycobacterium tuberculosis* (Black & DiRusso, 2003). In eukaryotic cells, Pat1p and Pat2p proteins are peroxisomal transmembrane proteins belonging to the ABC transporter superfamily involved in the uptake of LCFA for entry into the β-oxidation pathway at peroxisomes in *Saccharomyces cerevisiae* (Hettema *et al.*, 1996).

ABC transporters have been proposed to serve also as regulators of lipid metabolism in cells that can potentially affect the distribution of lipids directly or indirectly through the regulation of other proteins (Pohl *et al.*, 2005). In addition, some eukaryotic ABC transporters are involved in intracellular lipid homeostasis not only as they allow a precise balance between synthesis and uptake/efflux of metabolites, but also because certain defects of transporters cause important diseases due to disorders of lipid metabolism (Dean *et al.*, 2001). For these reasons, we analysed the potential relationship of Ltp1 with TAG metabolism in strain RHA1. In this context, we found that the overexpression of *ltp1* in strain RHA1 led to a significant increase (up to threefold) in the TAG content in comparison with the WT strain when cells were grown on lipophilic carbon sources under nitrogen-limiting conditions. Similarly, Kim *et al.* (2013) reported that an ABC transporter mediating transport of fatty acids from plastids to the endoplasmic reticulum in cells of *Arabidopsis thaliana* allowed enhanced TAG production in oleaginous seeds. In contrast to cells cultivated on lipophilic carbon sources, *ltp1*-overexpressing cells grown on gluconate produced decreased amounts of TAGs, but increased amounts of FFAs, compared with the WT (Fig. 3). These results suggested that Ltp1 may transport exogenous and also endogenous LCFAs, probably originating from lipid membrane turnover or remodelling and fatty acid recycling, affecting the distribution of the fatty acids into different metabolic pathways and lipid species. This fatty acid transporter might modulate, likely in concert with other proteins, the distribution of lipids between metabolic pathways, and may play a role in the exposure of fatty acids to acceptors involved in fatty acid and TAG metabolism in strain RHA1. The increase of TAG accumulation during growth of RHA1-pJAM2/*ltp1* cells on lipophilic carbon sources, the increase of the relative proportion of $C_{16:0}$ fatty acid in the TAG fraction produced by RHA1-pJAM2/*ltp1* during cultivation on palmitic acid as a carbon source and the increased production of TAGs by RHA1-pJAM2/*ltp1* seen using the fluorescently labelled fatty acid for cultivation suggested that Ltp1 may provide fatty acids to the three different acyltransferases involved in the Kennedy pathway for the biosynthesis of TAGs in strain RHA1 (Fig. 5). This process may occur close to the inner face of membrane, where the wax esters/DAG acyltransferases are located (Wältermann & Steinbüchel, 2005). The increasing number of transporter proteins attached to membranes in strain RHA1 overexpressing *ltp1* is likely to lead to deregulated fatty acid fluxes and disruption of lipid homeostasis (to some extent) during the growth of cells on gluconate as the sole carbon source under lipid accumulation conditions, as suggested by the increase of FFAs and the slight decrease of TAG content in recombinant cells. Several attempts to delete *ltp1* were made in this study, but we failed to generate the corresponding mutant. This result could suggest that the deletion of *ltp1* may be critical for survival of RHA1 cells, at least under the conditions used in this study. Rhodococci differ radically from other bacteria in the high content and large variety of lipids found in their different cellular structures (e.g. cell envelope, inclusion bodies, etc.). In this context, a large portion of the *R. jostii* RHA1 genome codes for enzymes involved in lipid metabolism (McLeod *et al.*, 2006). Fatty acids are probably key intermediates in rhodococci for the biosynthesis of many of the lipid species which perform important functions in their interaction with the environment (Fig. 5). In this context, the Ltp1 transporter may contribute to fatty acid recycling, management of membrane and envelope lipids, and maintenance of internal fatty acids pools for the production of a large variety of lipids of different complexity and function. For these reasons, the deletion of *ltp1* may disturb the dynamics of the lipid machinery affecting cell survival under certain circumstances.

In summary, in this study we report, for first time to our knowledge, a half-sized ABC transporter in the oleaginous *R. jostii* RHA1, which mediates exogenous and probably also endogenous LCFA import, and provides fatty acids for acyltransferase enzymes involved in TAG biosynthesis and probably other lipid pathways. This transport protein, which is only present in oleaginous rhodococci, may help to modulate the fatty acid distribution between lipid metabolic pathways affecting the lipid homeostasis of cells.

Insights into the function of membrane-embedded transporter proteins are also relevant for biotechnological

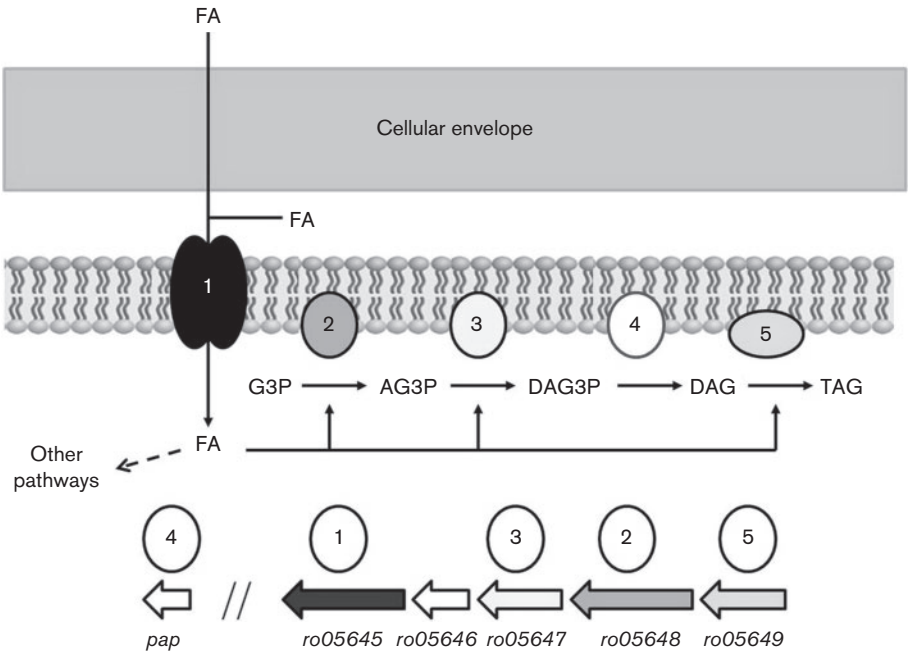

Fig. 5. Integrated overview of Ltp1 and the putative proteins involved in TAG metabolism, and organization of the genes coding for these proteins. FA, fatty acid; G3P, *sn*-glycerol 3-phosphate; AG3P, acylglycerol 3-phosphate; DAG3P, DAG 3-phosphate; *pap*, phosphatidic acid phosphatase gene.

purposes (Doshi *et al.*, 2013). The engineering of micro-organisms to produce commercial oils or biofuels is an interesting avenue (Alvarez & Steinbüchel, 2010; Holder *et al.*, 2011). This study showed the potential of expressing the identified transporter protein in strain RHA1 or in other oleaginous rhodococci to achieve efficiently the import of fatty acid-rich industrial wastes.

ACKNOWLEDGEMENTS

We thank Hugo Gramajo for reading the manuscript and his critical opinions. The technical assistance of Isella Gutierrez for the fluorescently labelled fatty acid measurements and of Enrique Rost for GC analyses is gratefully acknowledged. We also acknowledge Alberto Iglesias and Sergio Guerrero for their collaboration to this work. Martin Hernández provided strain RHA1/pJAM2 for this study, which was constructed for a previously unpublished work. H. M. A. is a Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. This study was financially supported by the SCyT of the University of Patagonia San Juan Bosco, the Agencia Comodoro Conocimiento (MCR), Oil m&s Company, CONICET (Project PIP-CONICET no. 0764), COFECyT (Project PFIP CHU-25) and ANPCyT (Project PICT2012 no. 2031), Argentina.

REFERENCES

Alvarez, H. M. & Steinbüchel, A. (2002). Triacylglycerols in prokaryotic microorganisms. *Appl Microbiol Biotechnol* **60**, 367–376.

Alvarez, H. M. & Steinbüchel, A. (2010). Physiology, bio-chemistry and molecular biology of triacylglycerol accumulation by *Rhodococcus*. In *Biology of Rhodococcus* (Microbiology Monographs, vol. 16), pp. 263–290. Edited by H. M. Alvarez & A. Steinbüchel. Heidelberg: Springer.

Alvarez, H. M., Mayer, F., Fabritius, D. & Steinbüchel, A. (1996). Formation of intracytoplasmic lipid inclusions by *Rhodococcus opacus* strain PD630. *Arch Microbiol* **165**, 377–386.

Alvarez, A. F., Alvarez, H. M., Kalscheuer, R., Wältermann, M. & Steinbüchel, A. (2008). Cloning and characterization of a gene involved in triacylglycerol biosynthesis and identification of additional homologous genes in the oleaginous bacterium *Rhodococcus opacus* PD630. *Microbiology* **154**, 2327–2335.

Alvarez, H. M., Silva, R. A., Herrero, M., Hernández, A. M. & Villalba, M. S. (2013). Metabolism of triacylglycerols in *Rhodococcus* species: insights from physiology and molecular genetics. *J Mol Biochem* **2**, 69–78.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M. & other authors (2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* **9**, 75.

Black, P. N. & DiRusso, C. C. (2003). Transmembrane movement of exogenous long-chain fatty acids: proteins, enzymes, and vectorial esterification. *Microbiol Mol Biol Rev* **67**, 454–472.

Borst, P., Zelcer, N. & van Helvoort, A. (2000). ABC transporters in lipid transport. *Biochim Biophys Acta* **1486**, 128–144.

Brandl, H., Gross, R. A., Lenz, R. W. & Fuller, R. C. (1988). *Pseudomonas oleovorans* as a source of poly(hydroxyalkanoates) for potential applications as biodegradable polyesters. *Appl Environ Microbiol* **54**, 1977–1982.

Chen, Y., Ding, Y., Yang, L., Yu, J., Liu, G., Wang, X., Zhang, S., Yu, D., Song, L. & other authors (2013). Integrated omics study delineates

the dynamics of lipid droplets in *Rhodococcus opacus* PD630. *Nucleic Acids Res* **42**, 1052–1064.

Dassa, E. & Bouige, P. (2001). The ABC of ABCs: a phylogenetic and functional classification of ABC systems in living organisms. *Res Microbiol* **152**, 211–229.

Davidson, A. L. & Chen, J. (2004). ATP-binding cassette transporters in bacteria. *Annu Rev Biochem* **73**, 241–268.

Dean, M., Hamon, Y. & Chimini, G. (2001). The human ATP-binding cassette (ABC) transporter superfamily. *J Lipid Res* **42**, 1007–1017.

Ding, Y., Yang, L., Zhang, S., Wang, Y., Du, Y., Pu, J., Peng, G., Chen, Y., Zhang, H. & other authors (2012). Identification of the major functional proteins of prokaryotic lipid droplets. *J Lipid Res* **53**, 399–411.

Doshi, R., Nguyen, T. & Chang, G. (2013). Transporter-mediated biofuel secretion. *Proc Natl Acad Sci U S A* **110**, 7642–7647.

Eckford, P. D. W. & Sharom, F. J. (2008). Functional characterization of *Escherichia coli* MsbA: interaction with nucleotides and substrates. *J Biol Chem* **283**, 12840–12850.

Hara, H., Stewart, G. R. & Mohn, W. W. (2010). Involvement of a novel ABC transporter and monoalkyl phthalate ester hydrolase in phthalate ester catabolism by *Rhodococcus jostii* RHA1. *Appl Environ Microbiol* **76**, 1516–1523.

Hernández, M. A., Arabolaza, A., Rodríguez, E., Gramajo, H. & Alvarez, H. M. (2013). The *atf2* gene is involved in triacylglycerol biosynthesis and accumulation in the oleaginous *Rhodococcus opacus* PD630. *Appl Microbiol Biotechnol* **97**, 2119–2130.

Hettema, E. H., van Roermund, C. W. T., Distel, B., van den Berg, M., Vilela, C., Rodrigues-Pousada, C., Wanders, R. J. & Tabak, H. F. (1996). The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of *Saccharomyces cerevisiae*. *EMBO J* **15**, 3813–3822.

Holder, J. W., Ulrich, J. C., DeBono, A. C., Godfrey, P. A., Desjardins, C. A., Zucker, J., Zeng, Q., Leach, A. L. B., Ghiviriga, I. & other authors (2011). Comparative and functional genomics of *Rhodococcus opacus* PD630 for biofuels development. *PLoS Genet* **7**, e1002219.

Jorgensen, J. H. & Ferraro, M. J. (2009). Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin Infect Dis* **49**, 1749–1755.

Kalscheuer, R., Arenskötter, M. & Steinbüchel, A. (1999). Establishment of a gene transfer system for *Rhodococcus opacus* PD630 based on electroporation and its application for recombinant biosynthesis of poly(3-hydroxyalkanoic acids). *Appl Microbiol Biotechnol* **52**, 508–515.

Kaul, G. & Pattan, G. (2011). MsbA ATP-binding cassette (ABC) transporter of *E. coli*: structure and possible flippase mechanism. *Indian J Biochem Biophys* **48**, 7–13.

Kim, S., Yamaoka, Y., Ono, H., Kim, H., Shim, D., Maeshima, M., Martinoia, E., Cahoon, E. B., Nishida, I. & Lee, Y. (2013). AtABCA9 transporter supplies fatty acids for lipid synthesis to the endoplasmic reticulum. *Proc Natl Acad Sci U S A* **110**, 773–778.

LeBlanc, J. C., Gonçalves, E. R. & Mohn, W. W. (2008). Global response to desiccation stress in the soil actinomycete *Rhodococcus jostii* RHA1. *Appl Environ Microbiol* **74**, 2627–2636.

MacEachran, D. P. & Sinskey, A. J. (2013). The *Rhodococcus opacus* TadD protein mediates triacylgylycerol metabolism by regulating intracellular NAD(P)H pools. *Microb Cell Fact* **12**, 104.

MacEachran, D. P., Prophete, M. E. & Sinskey, A. J. (2010). The *Rhodococcus opacus* PD630 heparin-binding hemagglutinin homolog TadA mediates lipid body formation. *Appl Environ Microbiol* **76**, 7217–7225.

Maloy, S. R., Ginsburgh, C. L., Simons, R. W. & Nunn, W. D. (1981). Transport of long and medium chain fatty acids by *Escherichia coli* K12. *J Biol Chem* **256**, 3735–3742.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Marqués, A. M., Pinazo, A., Farfan, M., Aranda, F. J., Teruel, J. A., Ortiz, A., Manresa, A. & Espuny, M. J. (2009). The physicochemical properties and chemical composition of trehalose lipids produced by *Rhodococcus erythropolis* 51T7. *Chem Phys Lipids* **158**, 110–117.

McLeod, M. P., Warren, R. L., Hsiao, W. W. L., Araki, N., Myhre, M., Fernandes, C., Miyazawa, D., Wong, W., Lillquist, A. L. & other authors (2006). The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. *Proc Natl Acad Sci U S A* **103**, 15582–15587.

Nagao, K., Kimura, Y., Mastuo, M. & Ueda, K. (2010). Lipid outward translocation by ABC proteins. *FEBS Lett* **584**, 2717–2723.

Pohl, A., Devaux, P. F. & Herrmann, A. (2005). Function of prokaryotic and eukaryotic ABC proteins in lipid transport. *Biochim Biophys Acta* **1733**, 29–52.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schlegel, H. G., Kaltwasser, H. & Gottschalk, G. (1961). [A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies]. *Arch Mikrobiol* **38**, 209–222 (in German).

Schneider, E. & Hunke, S. (1998). ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiol Rev* **22**, 1–20.

Swain, K., Casabon, I., Eltis, L. D. & Mohn, W. W. (2012). Two transporters essential for reassimilation of novel cholate metabolites by *Rhodococcus jostii* RHA1. *J Bacteriol* **194**, 6720–6727.

van der Geize, R., Hessels, G. I., van Gerwen, R., Vrijbloed, J. W., van der Meijden, P. & Dijkhuizen, L. (2000). Targeted disruption of the *kstD* gene encoding a 3-ketosteroid Δ^1-dehydrogenase isoenzyme of *Rhodococcus erythropolis* strain SQ1. *Appl Environ Microbiol* **66**, 2029–2036.

van Meer, G., Halter, D., Sprong, H., Somerharju, P. & Egmond, M. R. (2006). ABC lipid transporters: extruders, flippases, or flopless activators? *FEBS Lett* **580**, 1171–1177.

van Veen, H. W., Venema, K., Bolhuis, H., Oussenko, I., Kok, J., Poolman, B., Driessen, A. J. M. & Konings, W. N. (1996). Multidrug resistance mediated by a bacterial homolog of the human multidrug transporter MDR1. *Proc Natl Acad Sci U S A* **93**, 10668–10672.

Velamakanni, S., Yao, Y., Gutmann, D. A. P. & van Veen, H. W. (2008). Multidrug transport by the ABC transporter Sav1866 from *Staphylococcus aureus*. *Biochemistry* **47**, 9300–9308.

Villalba, M. S., Hernández, M. A., Silva, R. A. & Álvarez, H. M. (2013). Genome sequences of triacylglycerol metabolism in *Rhodococcus* as a platform for comparative genomics. *J Mol Biochem* **2**, 94–105.

Wältermann, M. & Steinbüchel, A. (2005). Neutral lipid bodies in prokaryotes: recent insights into structure, formation, and relationship to eukaryotic lipid depots. *J Bacteriol* **187**, 3607–3619.

Wältermann, M., Luftmann, H., Baumeister, D., Kalscheuer, R. & Steinbüchel, A. (2000). *Rhodococcus opacus* strain PD630 as a new source of high-value single-cell oil? Isolation and characterization of triacylglycerols and other storage lipids. *Microbiology* **146**, 1143–1149.

Warhurst, A. M. & Fewson, C. A. (1994). Biotransformations catalyzed by the genus *Rhodococcus*. *Crit Rev Biotechnol* **14**, 29–73.

Zhou, Z., White, K. A., Polissi, A., Georgopoulos, C. & Raetz, C. R. H. (1998). Function of *Escherichia coli* MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis. *J Biol Chem* **273**, 12466–12475.

Edited by: Y. Ohnishi